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Comparison of lipopolysaccharide and protein profiles between *Flavobacterium columnare* strains from different genomovars

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Abstract

Lipopolysaccharide (LPS) and total protein profiles from four *Flavobacterium columnare* isolates were compar. These strains belonged to genetically different groups and/or presented distinct virulence properties. *Flavobacterium columnare* isolates ALG-00-530 and ARS-1 are highly virulent strains that belong to different genomovars while *F. columnare* FC-RR is an attenuated mutant used as a live vaccine against *F. columnare*. Strain ALG-03-063 is included in the same genomovar group as FC-RR and presents a similar genomic fingerprint. Electrophoresis of LPS showed qualitative differences among the four strains. Further analysis of LPS by immunoblotting revealed that the avirulent mutant lacks the higher molecular bands in the LPS. Total protein analysis displayed by immunoblotting showed differences between the strains analysed although common bands were present in all the isolates. FC-RR lacked two distinct common bands (34 and 33 kDa) shared by the other three isolates. Based on the difference of LPS and total protein profiles, it is possible to discriminate the attenuated mutant FC-RR from other *F. columnare* strains.

Keywords: *Flavobacterium columnare*, genomovars, lipopolysaccharide, modified live vaccine, virulence, whole protein profiles.

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Introduction

Flavobacterium columnare is the causal agent of columnaris disease, one of the most important bacterial diseases of freshwater fish. This bacterium is distributed world wide in aquatic environments, affecting wild and cultured fish as well as ornamental fish (Austin & Austin 1999). *Flavobacterium columnare* is considered the second most important bacterial pathogen in commercial cultured channel catfish, *Ictalurus punctatus* (Rafinesque), in the southeastern USA, second only to *Edwardsiella ictaluri* (Wagner, Wise, Khoo & Terhune 2002). Direct losses due to *F. columnare* are estimated in excess of millions of dollars per year. Mortality rates of catfish populations in ponds can reach 50–60% and can be as high as 90% in tank-held catfish fingerlings (USDA 2003a,b).

Columnaris disease usually begins as an external infection of fins, body surface or gills. The fins become necrotic with greyish to white margins, and initial skin lesions appear as discrete bluish areas that evolve into depigmented necrotic lesions. Skin lesions may have yellowish mucoid material accompanied by mild inflammation. Lesions can develop exclusively on the gills, which usually results in subacute disease and mortality, as is typically the case in young fish (Plumb 1999).

Due to the ubiquitous presence of *F. columnare* in aquatic environments, eradication of the disease in fish farms is not likely to occur. Control and treatment of columnaris have primarily been directed towards the use of improved water-management practices to reduce physiological and environmental

stress in the fish. Recently, a modified live *F. columnare* vaccine has been developed using a rifampicin-resistant strategy (Shoemaker, Klesius & Evans 2005b). This methodology was previously used by Montaraz & Winter (1986) to generate a rough *Brucella abortus* strain, currently employed as the official vaccine for cattle brucellosis in the USA. The same strategy was used by Klesius & Shoemaker (1999) to create an *Edwardsiella ictaluri* rifampicin-resistant mutant patented as a modified live vaccine against enteric septicaemia of catfish (ESC) (AQUAVAC-ESC®; Intervet, Millsboro, DE, USA). Characterization of *B. abortus* and *E. ictaluri* rifampicin-resistant mutants revealed that lipopolysaccharide (LPS), a main virulence factor for Gram-negative bacteria, lacked the high molecular bands observed in virulent isolates (Klesius & Shoemaker 1999; Vemulapalli, McQuiston, Schurig, Sriranganathan, Halling & Boyle 1999; Arias, Shoemaker, Evans & Klesius 2003).

Flavobacterium columnare belongs to the Cytophaga–Flavobacterium–Bacteroid group and is phylogenetically distant from the better studied subclass Gammaproteobacteria, which contains major human and animal pathogens (including *Brucella* and *Edwardsiella*). To date, virulence factors in *F. columnare* are poorly characterized. Specifically, the role of LPS in columnaris pathogenicity has not been explored. The main objective of this study was to investigate whether an attenuated *F. columnare* mutant originated through a rifampicin-resistance strategy presented a modified LPS and a different total protein profile by comparison with virulent strains.

Materials and methods

Bacterial isolates and culturing

Four *F. columnare* strains, ALG-00-530, FC-RR, ARS-1 and ALG-03-063 were used in this study. The FC-RR strain is a rifampicin-resistant mutant, avirulent for catfish (Shoemaker *et al.* 2005b). This attenuated mutant has been licensed as a modified live vaccine by Intervet, Inc. and it is now undergoing field trials (Patent no.: US 6 881 412B1). Unfortunately, the original parent strain used to derive the avirulent mutant could not be included in this work as it lost viability during storage. Instead, other comparable (same geographical origin and source) virulent *F. columnare* strains were analysed. Isolate ALG-00-530 was obtained from diseased channel catfish at the Alabama Fish Farming Center, Greensboro, AL. The ARS-1 isolate was recovered from diseased channel catfish at the Aquatic Animal Health Research Unit, USDA-ARS, Auburn, AL. These two isolates have been demonstrated to be virulent for channel catfish (Shoemaker, Klesius, Lim & Yildirim 2003a; Welker, Shoemaker, Arias & Klesius 2005). *Flavobacterium columnare* ALG-03-063 was also included because it was isolated from diseased channel catfish and was the genetically most similar strain to the avirulent mutant FC-RR present in our collection.

Genetic fingerprinting

A previous study by Arias, Welker, Shoemaker, Abernathy & Klesius (2004) divided *F. columnare* strains into four well-defined genetic groups. All catfish isolates were grouped into three different genogroups (I–III; genogroup IV included only tilapia isolates). Strains ALG-00-530 and ARS-1 belong to genogroups I and II, respectively; while ALG-03-063 belongs to genogroup III. The avirulent mutant FC-RR was not included in that study but was fingerprinted in the present work. Amplified fragment length polymorphism (AFLP) patterns from the FC-RR mutant were obtained as described by Arias *et al.* (2004), added to the existing database and analysed.

Production of polyclonal antiserum against *Flavobacterium columnare*

Twelve channel catfish were randomly divided into three groups of four fish each. Two groups were used to produce antibodies (Ab) against the ALG-00-530 and FC-RR *F. columnare* strains (Ab-ALG-00-530 and Ab-FC-RR) while the third group served as a negative control. Antigen preparation was performed according to Arias *et al.* (2003) with the following modifications: cells were grown overnight in modified Shieh broth and control fish were also inoculated with Shieh broth. Three weeks after booster immunization, fish were bled and antiserum was used to probe Western blots (see below). In order to determine the relative titre of the sera, enzyme-linked immunosorbent assays (ELISA) were conducted according to Shoemaker, Shelby & Klesius (2003b). Serum from each individual fish was tested against each antigen dilution.

Lipopolysaccharides and total protein extraction

The four isolates were cultured in modified Shieh broth for 24 h at 28 °C. Three millilitres of broth was centrifuged at 3000 ***g*** for 15 min. Pelleted cells were resuspended in lysis buffer and proteins were extracted according to Arias, Verdonck, Swings, Aznar & Garay (1997a). Crude LPS was extracted following the phenol–water protocol described by Westphal & Jann (1965). After lyophilization, the LPS extract was diluted in sample buffer at a final concentration of 1 mg mL^{-1}. Aqueous and phenol LPS phases were stored at −80 °C until use.

Lipopolysaccharides and total protein analysis

Electrophoresis

Lipopolysaccharides from both phases were resuspended in sample treatment buffer and electrophoresed by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4% stacking gel and a 20% separating gel (Laemmli 1970). Gels were run at 15 mAmp for 90 min. Gels were silver stained following the manufacturer's instructions for the Bio-Rad Silver Stain kit (Bio-Rad, Hercules, CA, USA). Total protein electrophoresis followed the procedure described by Arias, Verdonck, Swings, Garay & Aznar (1997b). Protein concentration was measured using the Quick Start Bradford protein assay (Bio-Rad) and approximately 15 μg of total proteins was loaded per lane. Coomassie staining was used to visualize the protein bands following standard methods (Sambrook, Fritsch & Maniatis 1989).

Western blotting

Protein and LPS samples were analysed in duplicate. First, samples were resolved on 12% SDS-PAGE gel and then transferred to polyvinylidene difluoride membrane (Bio-Rad) at 100 V for 1 h. After blotting, one membrane was incubated with anti-ALG-00-530 serum while the second membrane was incubated with anti-FC-RR serum. After blocking for 30 min, 1:500 diluted polyclonal catfish serum was incubated with the membrane overnight followed by 1-h incubations with a monoclonal antibody (E-8) specific for channel catfish IgM (1:10) (Klesius 1990), and labelled with conjugated goat antimouse Ig (1:5000), followed by detection with Opti-4CN^{T}M (Bio-Rad).

Results

Amplified fragment length polymorphism analysis

Figure 1 shows the results of the cluster analysis of the AFLP patterns from the strains used in this study. Attenuated mutant FC-RR displayed a unique AFLP fingerprint although it clustered among other genogroup III strains. The AFLP profile of strain ALG-03-063 presented the highest per cent of similarity with that of the attenuated mutant. Based on this result, ALG-03-063 was selected for further comparisons as the most genetically similar strain to FC-RR present in our collection (that currently includes more than 150 *F. columnare* isolates; data not shown).

ELISA analysis

All fish immunized with *F. columnare* yielded positive titres. Control fish presented a very low cross-reactivity with the sonicated cells. All immunized fish produced strong immunity against both antigens. Although antisera from immunized fish reacted with both ALG-00-530 and FC-RR antigens, the corresponding antigen provided the highest titre (data not shown). Individual fish sera showing the highest titre (≥1/800) were chosen for immunoblotting analysis (fish no. 3 anti-ALG-00-530 and fish no. 1 anti-FC-RR, respectively).

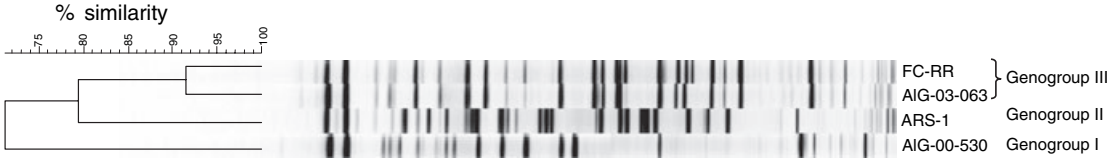

Figure 1 Dendrogram based on amplified fragment length polymorphism (AFLP) patterns of four strains of *Flavobacterium columnare* (ALG-00-530, FC-RR, ARS-1 and ALG-03-063). The tree was derived by UPGMA cluster analysis of the AFLP profiles.

Lipopolysaccharide characterization

The results of SDS-PAGE of LPS from *F. columnare* are shown in Fig. 2. The characteristic ladder-like pattern typical of Gram-negative pathogens was not observed. Only a few bands ranging from 3.5 to 17 kDa were present in all four strains. Phenol-phase extracts contained higher amounts of LPS than the aqueous-extracted samples. Strain ALG-00-530 showed a few bands in the phenol-phase extract while only one weak band was visualized in the aqueous phase. A similar pattern was observed with strain ARS-1 although band intensities were stronger. Attenuated mutant FC-RR shared a similar LPS pattern with ALG-03-063 although FC-RR bands were slightly smaller.

Bands from *F. columnare* LPS were visualized much more effectively in immunoblots. When catfish polyclonal antiserum was used for immunoblotting, important differences between the four strains were revealed (Fig. 3). While all strains isolated from diseased channel catfish (ALG-00-530, ARS-1 and ALG-03-063) presented high molecular bands (above 21 kDa) in both phenol and aqueous phases, the FC-RR mutant exhibited bands below that range. ALG-00-530 and ARS-1 again showed a similar LPS pattern regardless of antisera or extraction phase used. ALG-03-063 presented the same size bands in the phenol phase as FC-RR but also exhibited at least one higher molecular weight band that was absent in the mutant. Interestingly, these results were consistent regardless of antisera used (anti-ALG-00-530 or anti-FC-RR). However, significant differences between aqueous and phenol phase samples were found in both immunoblots.

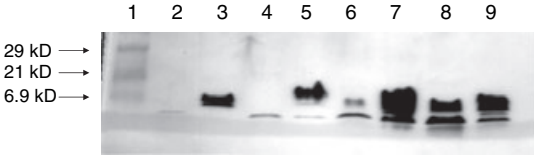

Figure 2 Silver stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis profiles of an lipopolysaccharide (LPS) preparation from *Flavobacterium columnare* strains used in this study. Lane 1, molecular standard; lanes 2–5, LPS aqueous phase from: ALG-00-530 (2), FC-RR (3), ARS-1 (4) and ALG-03-063 (5); lanes 6–9, LPS phenol phase from ALG-00-530 (6), FC-RR (7), ARS-1 (8) and ALG-03-063 (9).

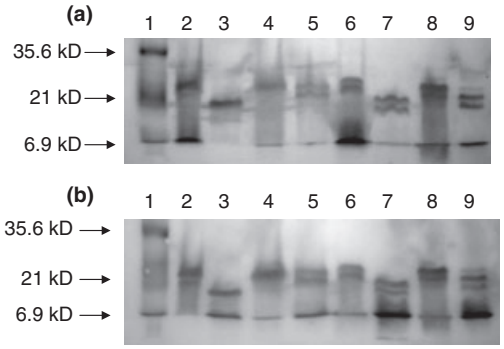

Figure 3 Immunoblot using anti-ALG-00-530 serum (a) and anti-FC-RR serum (b) with lipopolysaccharide (LPS) preparation. Lane 1, molecular standard; lanes 2–5, LPS aqueous phase from: ALG-00-530 (2), FC-RR (3), ARS-1 (4) and ALG-03-063 (5); lanes 6–9, LPS phenol phase from ALG-00-530 (6), FC-RR (7), ARS-1 (8) and ALG-03-063 (9).

Total protein analysis

Western blots of total proteins were also analysed with both sera (Fig. 4). The immunoblots indicated that the four strains did contain different antigenic proteins but also shared some common bands. ARS-1 showed more distinct antigenic protein bands than any other strain analysed (Fig. 4). Strains ALG-00-530, ARS-1 and ALG-03-063 contained two very distinct bands around 35 kDa. The FC-RR mutant lacked these bands but instead presented two additional bands in the 30 kDa range. Each strain had a very similar banding pattern with anti-ALG-00-530 and anti-FC-RR sera. When ALG-530 serum was used, reactivity against low molecular weight bands present in the three clinical strains was observed. FC-RR low molecular bands did not react with anti-ALG-00-530 serum. On the contrary, when anti-FC-RR was used, low molecular weight proteins in FC-RR were revealed.

Discussion

Lipopolysaccharide is a major suprastructure of Gram-negative bacteria which contributes greatly to the structural integrity of the bacteria, and protects them from host immune defences. The LPS has been described and well characterized as a virulence factor in many bacterial species, e.g. *Salmonella* sp. (Makela, Valtonen & Valtonen 1973), *Escherichia coli* (Medearis, Camitta & Heath 1968), *Shigella flexneri* (Rajakumar, Jost, Sasakawa, Okada, Yoshikawa & Adler 1994), *Brucella abortus* (Vemulapalli, He, Buccolo, Boyle, Sriranganathan & Schurig

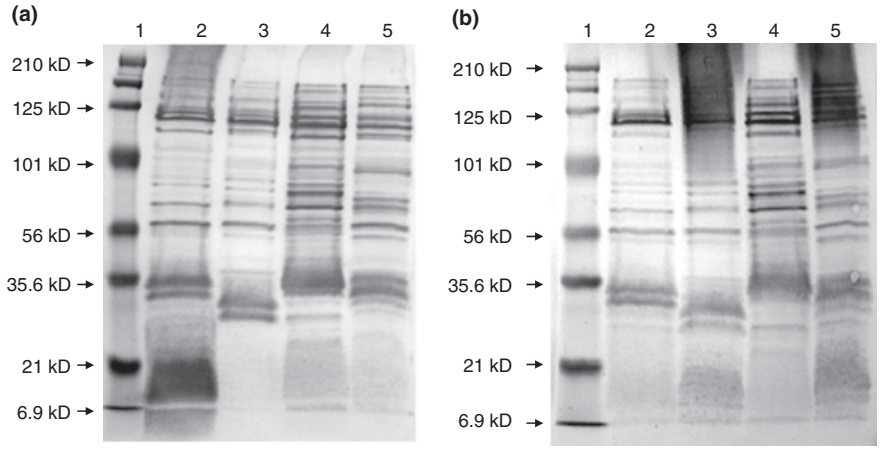

Figure 4 Western blot analysis of total protein with anti-ALG-00-530 serum (a) and anti-FC-RR serum (b). Lane 1, molecular standard; lane 2, ALG-00-530; lane 3, FC-RR; lane 4, ARS-1; lane 5, ALG-03-063.

2000) and including fish pathogens such as *Vibrio vulnificus* (Amaro, Fouz, Biosca, Marco-Noales & Collado 1997), *E. ictaluri* (Arias *et al.* 2003) and *F. psychrophilum* (MacLean, Vinogradov, Crump, Perry & Kay 2001). Attempts to characterize the structure of the LPS in *F. columnare* were made by MacLean, Perry, Crump & Kay (2003) and by Vinogradov, Perry & Kay (2003). Unfortunately, the strain used by these authors (ATCC 43622) had been originally misidentified as *F. columnare* but actually belongs to the species *Flavobacterium johnsoniae* (Darwish, Ismaiel, Newton & Tang 2004; Shoemaker, Arias, Klesius & Welker 2005a). Therefore, no information on *F. columnare* LPS composition or immunogenic role was available.

In this study, catfish polyclonal antisera against two isolates, ALG-00-530 and FC-RR (attenuated mutant) were generated and used to determine LPS immunogenic properties between genetically different *F. columnare* strains. Differences in LPS between four isolates of *F. columnare* were evident after electrophoresis and silver staining, but were even more obvious after Western blot analysis. Although a typical LPS ladder has been reported in other *Flavobacterium* species (MacLean *et al.* 2001), our silver staining of *F. columnare* LPS showed only a few bands.

Immunogenic bands present in the LPS were revealed by immunoblots. However, there were differences in band molecular weights between strains isolated from diseased channel catfish and the attenuated strain FC-RR. The three isolates from diseased fish (ALG-00-530, ARS-1 and ALG-03-063) presented LPS bands between 21 and 29 kDa while FC-RR exhibited a unique band under 21 kDa. Our results suggest that the induction of rifampicin-resistant mutation(s) in *F. columnare* results in loss of the high molecular weight bands displayed by virulent isolates. Similar results were reported in *B. abortus* and *E. ictaluri* (Arias *et al.* 2003) when LPS from rifampicin-resistant mutants was analysed. Rifampicin is a potent and broad-spectrum antibiotic well known as a DNA-directed RNA polymerase inhibitor. Mutations in the RNA polymerase gene that conferred resistance against rifampicin have been widely documented (Jin & Gross 1988). On the other hand, the mechanism by which rifampicin induces mutations that affect LPS structure are relatively poorly known. It is remarkable how such different pathogens (*Brucella*, *Edwardsiella* and *Flavobacterium*) behave similarly under the same stressor suggesting that short O-chain LPS will favour cell survival. It has been postulated (Kirschbaum & Gotte 1993) that rifampicin, like other hydrophobic molecules, enters the cell via simple diffusion through the outer membrane with LPS being the main obstacle. A structural change in the LPS might enhance the barrier effect protecting the cell against rifampicin. Vemulapalli *et al.* (1999) suggested that multiple genes involved in LPS biosynthesis are disrupted in *B. abortus* and this results in the attenuated phenotype.

Our results indicate that the LPS in *F. columnare* may play an important role in columnaris pathogenesis as a lack of LPS high molecular weight bands seems to be correlated with total lack of virulence in other bacterial species (Kimura &

Hansen 1986; Rajakumar *et al.* 1994; Amaro *et al.* 1997; Arias *et al.* 2003). Although a thorough antigenic characterization in *F. columnare* has not yet been attempted, some serological diversity has been reported between isolates (Anacker & Ordal 1959; Sanders, Holt & Fryer 1976). This antigenic variation can be justified by the genetic variability observed in this species (Arias *et al.* 2003; Thomas-Jinu & Goodwin 2004). In fact, we observed some serological differences in LPS within the virulent strains. While ALG-00-530 and ARS-1 both showed a very similar pattern denoting common antigens, ALG-03-063 presented at least one additional LPS band regardless of extraction phase and antisera used. Interestingly, these three strains belong to different genogroups and present very distinct fingerprinting profiles; however, ALG-00-530 and ARS-1 showed very similar results with LPS immunoblots. These two strains have been used by our group in virulence studies and although both are able to cause columnaris disease in catfish by immersion, ALG-00-530 has consistently exhibited a higher mortality rate than ARS-1 (C. D. Shoemaker, unpublished data).

When we compared the antigenic variability exhibited by the total protein profiles, all four strains showed a different pattern. Differences in protein composition between isolates were expected as they presented differences in their genome. However, there was a large set of common bands shared by all strains and revealed by both antisera. This indicates that the FC-RR mutant presents a number of common epitopes with other *F. columnare* isolates and could explain why this strain used as a modified live vaccine is able to confer protection against different *F. columnare* isolates.

In conclusion, we have shown that the *F. columnare* FC-RR isolate lacks the high molecular weight components of LPS present in other virulent isolates. Differences in LPS and immunogenic proteins were observed between genetically different *F. columnare* isolates. A secondary outcome of this study was the genetic fingerprinting of FC-RR. The attenuated mutant presents a unique AFLP profile, not present in our existing database, which can be used to track the modified live vaccine strain (FC-RR) in the fish farm environment.

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